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Development of an FHbp-CTB holotoxin-like chimera and the elicitation of bactericidal antibodies against serogroup B *Neisseria meningitidis*

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ABSTRACT

The *Neisseria meningitidis* factor H binding protein (FHbp) is an important virulence factor and vaccine antigen contained in both USA licensed serogroup B meningococcal vaccines. Recent studies in human factor H (hFH) transgenic mice suggest that hFH-FHbp interactions lower FHbp-elicited immunogenicity. To provide tools with which to characterize and potentially improve FHbp immunogenicity, we developed an FHbp-cholera holotoxin-like chimera vaccine expression system in *Escherichia coli* that utilizes cholera toxin B (CTB) as both a scaffold and adjuvant for FHbp. We developed FHbp–CTB chimeras using a wild-type (WT) FHbp and a low hFH-binding FHbp mutant R41S. Both chimeras bound to G_{M1} ganglioside and were recognized by the FHbp-specific monoclonal antibody JAR4. The R41S mutant had greatly reduced hFH binding compared to the WT FHbp-CTB chimera. WT and R41S FHbp-CTB chimeric antigens were compared to equimolar amounts of FHbp admixed with CTB or FHbp alone in mouse immunogenicity studies. The chimeras were significantly more immunogenic than FHbp alone or mixed with CTB, and elicited bactericidal antibodies against a panel of MenB isolates. This study demonstrates a unique and simple method for studying FHbp immunogenicity. The chimeric approach may facilitate studies of other protein-based antigens targeting pathogenic *Neisseria* and lay groundwork for the development of new protein based vaccines against meningococcal and gonococcal disease.

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1. Introduction

The *Neisseria meningitidis* factor H binding protein (FHbp) is a lipidated outer membrane protein and an important meningococcal virulence factor [1]. FHbp plays a critical role in serum resistance by binding to the human complement regulatory protein factor H (hFH) and downregulating the complement cascade [2,3]. In addition, studies have demonstrated knocking out FHbp expression can eliminate complement resistance of *N. meningitidis* [2,4]. Because of its importance as a meningococcal virulence factor and its ability to elicit protective immune responses in animal studies, FHbp was developed as a vaccine antigen and is a component of both currently licensed MenB vaccines [1,5–7].

Two different classification systems have been developed for FHbp based on amino acid sequence homology. Sequence types are divided into three variant groups (var1, var2, and var3) or two subfamilies (A and B) [5,6]. Subfamily A corresponds to var2 and var3, and subfamily B corresponds to var1. Unique sequences within a subfamily or variant group are given an identifying number. Immunization with FHbp can elicit cross-protective bactericidal antibodies to strains expressing FHbp within the same subfamily [5,6,8,9]. The cross-reactivity between var2 and var3 but not var1 is consistent with the data for the subfamilies [6,9]. In the USA, MenB strains expressing subfamily B are more common (59%) than subfamily A (41%) [10].

Two MenB vaccines have been approved for use in individuals 10 through 25 years of age in the USA [11,12]. One vaccine, Bexero® (4CMenB), contains three components in addition to recombinant FHbp: recombinant neisserial adhesion A (NadA), recombinant neisserial heparin binding protein (NHBP), and outer membrane vesicles (OMV) from strain NZ98/254 that include a porin protein PorA (serosubtype P1.4) [12]. FHbp (1.1; B24) is an important component of 4CMenB as adsorption of anti-FHbp IgG from human immune sera significantly reduces bactericidal titers against strains expressing homologous and heterologous FHbp [13,14]. The other licensed vaccine, Trumenba® (rLP2086), contains two lipidated recombinant FHbp antigens, one from subfamily A and one from subfamily B [15]. In clinical studies, both vaccines elicited bactericidal antibodies against selected MenB strains

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measured with the human complement serum bactericidal activity (hSBA) assay [15,16].

Although FHbp has been demonstrated to be an important protective antigen, it is unknown the extent to which FHbp interacts with FH upon immunization, and whether any FHbp-FH interaction is detrimental to the overall immunogenicity of FHbp. Studies in hFH transgenic mice and infant rhesus macaques, the latter having a polymorphism in the FH gene that allows for either high or low binding to FHbp [17], have revealed that binding of FH to FHbp lowers the immunogenicity of FHbp [18–22]. Furthermore, 10 distinct human anti-FHbp antibody fragments (Fabs), and affinity purified FHbp-specific antibodies obtained from individuals immunized with FHbp were demonstrated to be poor at blocking FH binding to the surface of MenB [23]. Although these antibodies were bactericidal, this study suggested a potential FHbp-FH interaction that skews the antibody repertoire to epitopes outside of the FHbp-FH binding pocket [23]. Subsequently, studies in both hFH transgenic mice and rhesus macaques determined that FHbp with mutations that significantly reduce FHbp-FH binding are more immunogenic and elicit greater bactericidal killing [19,22,24]. Therefore, studies of wild type and mutant FHbp antigens with diminished or abrogated hFH binding are warranted to expand our understanding of cross-protective responses and may ultimately lead to the development of more immunogenic and protective vaccines.

To further evaluate the immunogenicity of FHbp, we sought to develop a simple yet efficient method for recombinant production of soluble and immunogenic FHbp. In Trumenba®, lipidated FHbp is highly immunogenic likely due to the ability of the lipid tail to stimulate the pattern recognition receptor TLR2 [25]. However, expression of lipoproteins in *E. coli* can be challenging due to low protein expression and/or incomplete protein lipidation [26]. The FHbp contained in Bexsero® is an N-terminal fusion to GNA2091, a periplasmic lipoprotein that may stabilize FHbp and potentially increase FHbp immunogenicity [27,28]. Here, we developed a novel approach utilizing FHbp-based cholera toxin holotoxin-like chimeras [29,30]. This method uses the natural non-covalent assembly of antigens to the non-toxic cholera toxin B subunit (CTB) via genetic fusions of antigen to the non-enzymatic cholera toxin A2 subunit [29,30]. CTB is a strong adjuvant and studies have indicated CTB adjuvanticity is enhanced if antigen and CTB are physically coupled [31]. We utilized a dual promoter expression plasmid previously described, with one promoter controlling expression of the *fhbp* inserted upstream and in-frame with the *a2* subunit of cholera toxin, and the second promoter controlling expression of CTB. Both FHbp-A2 and CTB contained signal sequences for periplasmic export where non-covalent assembly of FHbp to CTB (FHbp-CTB) occurs [32]. Here we demonstrate the production, characterization, and immunogenicity of two chimeras, FHbp-CTB and FHbpR41S-CTB, the latter containing a mutation that markedly diminishes hFH binding to FHbp [18].

2. Methods

2.1. Construction of recombinant protein expression plasmids

The expression plasmid used to create the FHbp holotoxin-like chimera was designated pGAP22 and is a derivative of a previously published dual promoter expression plasmid, pGAP22A2 [32]. Both pGAP22 and pGAP22A2 are identical in design and restriction sites except pGAP22 contains a shortened A2 domain starting at amino acid 211 (Leu) of the cholera toxin A subunit [33]. The *fhbp* gene was PCR amplified from genomic DNA obtained from MenB strain Cu385 (FHbp 1.1/B24) [34]. The forward and reverse primers contained MscI and NotI restriction sites, respectively (Table 1). The forward primer started at amino acid 13 of the mature FHbp protein and the reverse primer started at the final amino acid of FHbp. The resultant PCR amplified *fhbp* gene was cloned into the MscI and NotI sites of pGAP22, which sandwiched it in-frame between the *pelB* leader sequence and the shortened *a2* domain of cholera toxin. The new plasmid, pGAP22-FHbp, contained an IPTG-inducible T7 promoter controlling expression of the *fhbp-a2* gene product, and an arabinose inducible promoter controlling the expression of the *ctb* gene product [32].

To create the FHbpR41S low hFH-binding mutant holotoxin-like chimera the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) was used following the manufacturer's protocol. Mutagenic primers (Table 1) were used to mutagenize pGAP22-FHbp to create the new plasmid pGAP22-FHbpR41S. To make recombinant FHbp, the gene was PCR amplified from genomic Cu385 DNA as above and the gene product was inserted into the NdeI and XhoI restriction sites of the expression plasmid pET28 (EMD Millipore) and was in frame with both an N- and C-terminal 6-histidine tag. CTB was made using the CTB expression plasmid previously described [32].

2.2. Protein expression and purification

All recombinant proteins were expressed under the same conditions but utilized different expression strains. Both the FHbp-CTB and FHbpR41S-CTB variants were expressed in BL21 Star™ (DE3) (Thermo Fisher Scientific), and CTB in BL21 (DE3). Recombinant FHbp was expressed in SHuffle Express® (DE3) (New England Biolabs). For protein expression, strains were grown in half-liter volumes in NZTCYM (1% NZ-amine, 1% tryptone, 0.5% NaCl, 0.5% yeast extract, 0.1% casamino acids, 0.2% $MgSO_4$) pH 7.5 with 50 μg/mL of kanamycin [35]. Cultures were grown at 37 or 30 °C (SHuffle Express®) and 250 rpm until they reached an OD_{600} of approximately 3.0. The cultures were then grown at 16 °C and 250 rpm for approximately 30 min to acclimate to the new temperature then induced. For both the FHbp-CTB and FHbpR41S-CTB variants the cultures were induced with 0.1 mM IPTG and 0.1% arabinose. For both CTB and FHbp, expression was induced

Table 1
Primers used for making expression plasmids.

Primer	Sequence
FHbp-Forward-MscI	ACTTGGCCACAGGTGCGGGGCTTGCCGATGC
FHbp-Reverse-NotI	TTGCGGCCGCTTGCTTGGCGGCAAGGCCGATATG
FHbpR41S-mutation-For	GCTGGATCAGTCCGTCAGCAAAAACGAGAAACTGAAGCTGG
FHbpR41S-mutation-Rev	CCAGCTTCAGTTTCTCGTTTTTGCTGACGGACTGATCCAGC
FHbp-Forward-NdeI	ATCATATGGGTGCGGGGCTTGCCGATGC
FHbp-Reverse-XhoI	ATTCACTCGAGTTGCTTGGCGGCAAGGCCGATATG

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